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A novel multi-pad paper plate (MP^3) based assays for rapid animal disease diagnostics

Valentina Busin [a,b,*], Stewart Burgess[a], Wenmaio Shu[b,c]

a Moredun Research Institute, Pentlands Science Park, Bush Loan, Edinburgh EH26 0PZ, United Kingdom
b School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh EH14 4AS, United Kingdom
c Department of Biomedical Engineering, University of Strathclyde, Glasgow. G4 0NW. United Kingdom

Abstract

This study describes a novel fabrication method of a multi-pad paper plate (MP^3) for transfer and optimisation of enzyme-linked immunosorbent assays (ELISA) on paper. The plate was fabricated using a combination of laser cutting and lamination for rapid and low-cost production of a multi-pad paper plate compatible with a standard 96 well microplate format. The plate was used to transfer a sandwich ELISA assay for detection of the acute phase protein bovine haptoglobin, allowing assay optimisation in the new format. The optimised protocol was also applied to detection of haptoglobin in bovine serum, demonstrating significant correlation with the traditional lab-based ELISA.

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Keywords: P-ELISA; microfluidics; veterinary medicine; haptoglobin; point-of-care

1. Introduction

In the fast growing field of microfluidic paper-based analytical devices (μPADs), an interesting application has recently been demonstrated in the translation of ELISA onto paper or P-ELISA [1, 2]. P-ELISA combines the sensitivity and specificity of ELISA with the intrinsic low cost and ease-of-use of paper-based platforms. The most commonly used methods for fabrication of these platforms are photolithography [3] and wax printing techniques [4].

* Corresponding author. Tel.: +44-0131-445-7452.
E-mail address: valentina.busin@moredun.ac.uk

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However, both methods require specialised machinery (wax printer) or chemical substances (e.g. SU-8 photoresist) to create hydrophobic barriers. An alternative method, which is both simpler and cheaper but still allows for high throughput, is the use of high precision laser micromachining techniques to create microfluidic patterns and lamination to control reactions [5]. In veterinary diagnostics, the use of haptoglobin (Hp) as a marker of disease status has recently attracted considerable attention [6-8]. Measurement of Hp in bovine serum is currently available with either a colorimetric reaction based on peroxidase activity of the haptoglobin-haemoglobin complex or with a commercially available ELISA kit [9]. Both assays are lab-based, with considerable time and costs involved. The possibility of detecting the level of Hp at the point-of-care using µPADs, therefore, has obvious benefits, allowing for disease to be diagnosed at the animal-side in a timely and cost-effective manner.

This study describes a novel fabrication method for realisation of a multi-pad paper plate (MP^3), which can be used to translate a lab-based ELISA assay into a P-ELISA. The manufacturing process is reported as well as the application of a sandwich ELISA for the detection of Hp in bovine serum.

2. MP^3 design and fabrication

The MP^3 template was designed using a 3D mechanical computer-aided design package (Solid Work 2012) and produced for both the paper and a complementary plastic lamination sheet for packaging (Fig. 1**Error! Reference source not found.**) using a class 2 CO2 laser (Speedy 300 Laser Engraver - Trotec) and a roll laminator (GBC Catena 35 Roll Laminator). The MP^3 consists of 96 circular paper pads of 6mm diameter, all completely independent from the others and compatible with instruments commonly used in laboratories for 96 well microplates. When tested with food colour, there was no evidence of cross-contaminations between pads, even when excess fluid (50µL) was deposited. A volume of 2µL was determined as the optimal amount to obtain complete wetting of the pads whilst minimising the drying time required at room temperature.

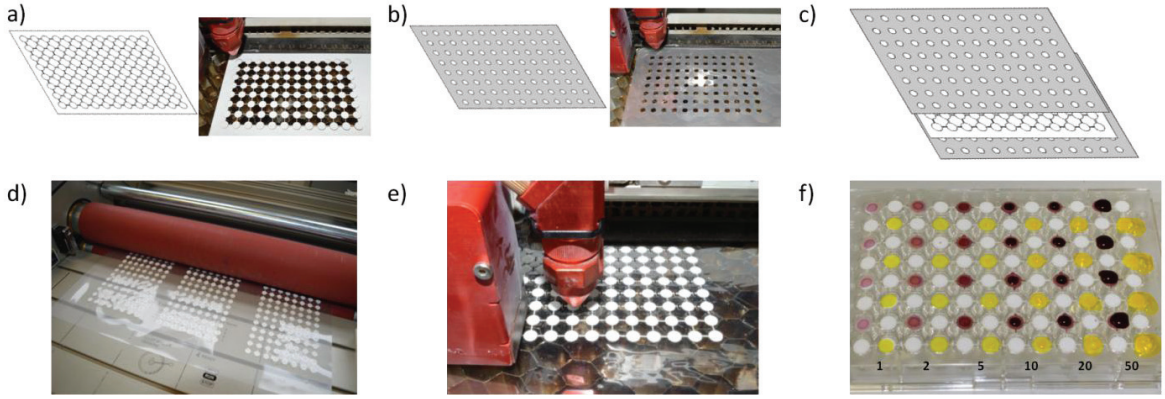

Fig. 1. Schematic of the fabrication of theMP^3. a) Whatman Grade 1 cellulose chromatography paper (Sigma-Aldrich, UK) cut with a laser cutter. b) 75µ gloss laminating pouches (Staples, UK) cut with a laser cutter. c) Assembly of device in the pre-cut lamination sheets. d) Lamination at 110°C at the lowest speed settings. e) Severing of interconnecting paper channels by laser cutter. f) Application of food colour to the device. Numbers represent volume of fluids applied (µl).

3. Sandwich Hp ELISA for animal diagnostics

A sandwich ELISA for the detection of bovine Hp (Fig. 2) was applied to the MP^3. Following image capture, mean gray intensity (mean pixel intensity) was measured with Image J by assessing signal measurement from each circular pad, following image inversion. All data were analyzed within GraphPad Prism 5 (GraphPad Software, Inc., USA). The standard curve was generated using non-linear regression and a sigmoidal curve response (variable slope) was obtained (Fig. 3). The limit of detection achieved was 0.73µg/ml (a commercially available lab-based ELISA kit can achieve approximately one order of magnitude lower).

Clinical bovine samples from 8 animals with different degrees of inflammation were also analysed on the MP^3 using the optimised sandwich Hp ELISA protocol and compared with results obtained from the same samples by a standard lab-based ELISA using Pearson correlation. A total of 8 serum samples were analysed in triplicates on two separate MP^3. There was a significant correlation ($p<0.05$) between results obtained from the lab-based ELISA and the protocol applied to the MP^3, when serum samples were diluted at 1:2 and 1:10 ($r= 0.73$ and $r= 0.85$ respectively).

When a cost-benefit analysis was run to compare the Hp ELISA performed on the MP^3 and the assay in the conventional format, a reduction of 88% in cost and 93% in assay time was achieved (Table 1).

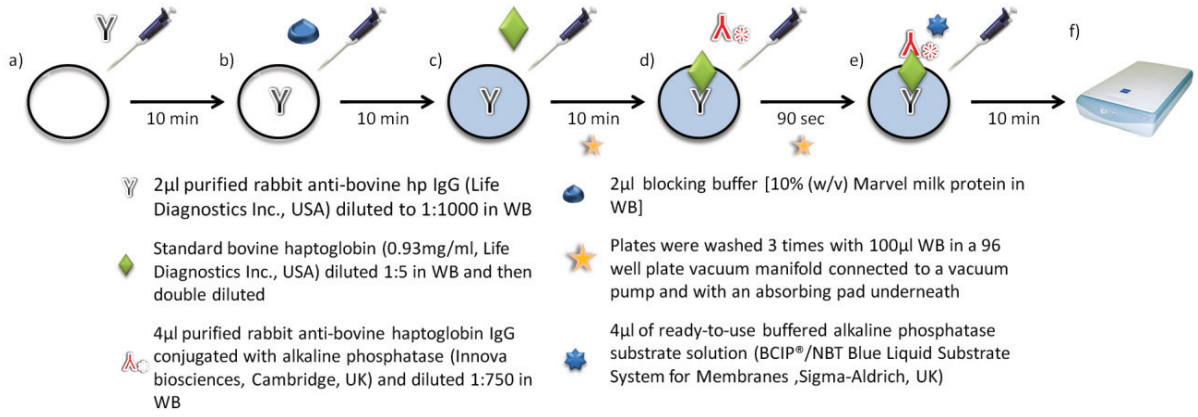

Fig. 2. Schematic representation of the sandwich ELISA for the detection of bovine Hp as applied to the MP^3 platform. a) Coating of the MP^3 with capture antibodies. b) Blocking to prevent non-specific binding. c) Addition of sample. d) Addition of conjugate. e) Addition of substrate. f) MP^3 scanned using a flatbed desktop scanner (Perfection V350 photo, Epson, UK, set to "color photo scanning", 1200 dpi resolution) and images transformed in 8-bit greyscale with PixBuilder Studio 2.2 (WnSoft Ltd., UK). All steps of the assay were performed at room temperature. WB: (0.02M Tris-HCl with 0.05% Tween-20, pH 7.4)].

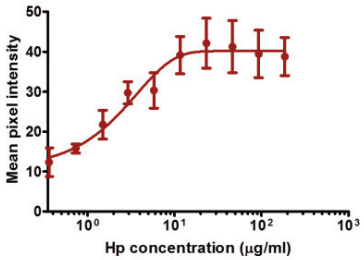

Fig. 3. Non linear regression (variable slope) of mean pixel intensity in relation to bovine standard Hp concentration (µg/ml). Each data point represents the mean of 12 replicate measurements, shown as 10 logarithmic conversion of Hp concentration. Error bars represent standard deviation

Table 1. Cost-benefit analysis of performing a sandwich Hp ELISA on MP^3 or with the conventional lab-based technique. * negligible cost.

	Hp ELISA on MP^3			Conventional Hp ELISA		
	Volume (µL)	**Time (min)**	**Cost per plate**	**Volume (µL)**	**Time (min)**	**Cost per plate**
Primary antibody	2	10	£1	100	480	£5.5
Blocking buffer	2	10	*	200	60	*
Antigen (Hp)	2	10	£1.2	100	60	£15
Conjugated antibody	4	1.5	£3.4	100	60	£20
Substrate	4	10	£0.4	100	10	£10.5
Plate	-	-	£0.3	-	-	£1.5
Total	**8**	**41.5**	**£6.3**	**500**	**670**	**£53**

4. Conclusions

The novel fabrication method described here is rapid, low-cost and of high-throughput and can be used to translate virtually any ELISA into a P-ELISA. It also represents the first example of paper microfluidic technologies being applied to veterinary medicine for quantitative analysis of clinical samples and a starting point for achieving true animal-side testing.

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